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14. ABSTRACT

Tuberous sclerosis complex (TSC) is a genetic disorder associated with severe neurological symptoms including mental retardation, autism, infantile spasms, and epilepsy. Seizures occur in the majority of TSC patients and are often refractory to antiepileptic drugs. Cortical tubers, the primary neuropathology in these patients, were identified as epileptogenic in several clinical studies. However, the contribution of TSC gene inactivation to altered excitability (in the absence of tuber formation) is not fully understood. Here we used antisense morpholino oligonucleotides to knockdown TSC genes in developing zebrafish (Danio rerio). Using intact agar-immobilized zebrafish larvae and extracellular recording techniques, we present evidence for neuronal hyperexcitability following loss of Tsc1. Importantly, tsc1 morphants show no evidence of tuber formation in histological studies. In addition, rapamycin effectively inhibited target of rapamycin (TOR) kinase activity in zebrafish with no amelioration of the hyperexcitable phenotype. Our study demonstrates that Tsc1 knockdown in a relatively simple brain structure can result in hyperexcitability.

15. SUBJECT TERMS
TSC (Tuberous Sclerosis Complex); zebrafish; seizures; antisense morpholino oligonucleotides, rapamycin, hyperexcitability

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Introduction

Seizures occur in nearly 90% of TSC patients. While cortical tubers are believed to participate in seizure genesis, epilepsy (a condition of recurrent spontaneous seizures) has been reported in TSC patients and mouse models lacking tubers. Thus, a fundamental question is whether TSC gene mutation, in the absence of tuber formation, result in seizures. To address this question we proposed to (i) knockdown TSC gene function in larval zebrafish using morpholino-based technology and (ii) assess seizure susceptibility in zebrafish morphants with reduced TSC gene function. Proposed experiments were designed to test the hypothesis that loss of TSC gene function directly impacts neuronal/synaptic function resulting in hyperexcitability and seizures.

Body

Task #1: To use morpholino antisense oligonucleotides to knockdown TSC genes in zebrafish (**Aim I**). Scanning the zebrafish genome databank we identified two TSC1 zebrafish homologs corresponding to *TSC1a* and *TSC1b*. Morpholino oligonucleotides available from Gene Tools LLC were ordered for each homolog. We selected morpholino sequences based on design parameters according to the manufacturer's recommendations, namely 21-25 mer antisense oligonucleotides of ~50% G/C and A/T content with no predicted internal hairpin loops. Microinjection into single cell embryos at 0 to 40 minutes post-fertilization was performed on >1000 embryos. Western blot analysis for TSC1 (hamartin) and TSC2 (tuberin) proteins were used to confirm the efficacy of morpholino injections. Morpholino concentrations between 0.5 and 1 mM were effective concentrations were effective in knocking down protein expression at 3 days postfertilization (dpf). To control for non-specific morpholino effects miss-sense and vehicle injections were made.

Task #2: To assess seizure behavior/electrophysiology in TSC-deficient zebrafish (**Aim II**). Here morpholino-injected zebrafish were analyzed for the presence of spontaneous seizure-like behavior. Observational studies and computer-assisted locomotion tracking studies confirmed seizure-like behaviors in Tsc1a morphant zebrafish. We then proceeded with electrophysiology studies to confirm an electrographic component. In vivo extracellular recordings were obtained from zebrafish immobilized in agar (n = 39). Stimulation of the contralateral eye evoked multiple epileptiform-like field responses in the optic tectum in 80% of Tsc1a morphants tested; less than 30% of control fish exhibited multiple field responses. In normal bathing medium, high-amplitude ictal-like spontaneous discharge was consistently observed in Tsc1a morphant zebrafish at 3 dpf; ictal-like activity was smaller and less frequent in controls. Seizure-like activity was not reduced in Tsc1a morphants treated with an inhibitor of the mTOR signaling pathway e.g., rapamycin

Key research accomplishments

- These studies represent the first time that TSC gene function has been studied in zebrafish.
- Our findings demonstrate that reduced expression of Tsc1 can lead to seizure-like behavior and electrographic seizure activity in developing zebrafish.
- Rapamycin does not ameliorate the seizure phenotype observed in Tsc1a morphant zebrafish

Reportable outcomes

- *Abstracts:* 2005 Annual meeting of the American Epilepsy Society. Abs. #IW.07. “Morpholino knockdown of tuberous sclerosis genes in developing zebrafish”. M.R. Taylor & S.C. Baraban.

- *Manuscripts:* 2006 Submitted to Journal of Neurobiology. “Hyperexcitability following knockdown of tuberous sclerosis complex (Tsc1) in zebrafish. M.R. Taylor, E.P. Harrington-Walker, M.T. Dinday & S.C. Baraban

- This award also supported preliminary studies by a postdoctoral fellow in my laboratory (Michael Taylor) that formed part of his successful Ruth L. Kirschstein National Research Service Award, Individual Fellowship.

Conclusion

In conclusion, we demonstrated that loss of *tsc* gene function in developing zebrafish results in hyperexcitability (a hallmark feature of an epileptic brain) in the absence of tuber formation. In addition, we provide evidence that rapamycin may not reduce the abnormal electrical activity associated with TSC-related seizures. These studies provide a new model to test (i) potential treatments for TSC patients and (ii) underlying mechanisms leading to seizure generation in TSC.

References

None

Appendices

Taylor et al. manuscript. Figures included in the manuscript represent supporting data.

Hyperexcitability Following Knockdown of Tuberous Sclerosis Complex 1 (Tsc1) in Zebrafish

Running title: Zebrafish Model of TSC

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ABSTRACT

Tuberous sclerosis complex (TSC) is a genetic disorder associated with severe neurological symptoms including mental retardation, autism, infantile spasms, and epilepsy. Seizures occur in the majority of TSC patients and are often refractory to antiepileptic drugs. Cortical tubers, the primary neuropathology in these patients, were identified as epileptogenic in several clinical studies. However, the contribution of TSC gene inactivation to altered excitability (in the absence of tuber formation) is not fully understood. Here we used antisense morpholino oligonucleotides to knockdown TSC genes in developing zebrafish (*Danio rerio*). Using intact agar-immobilized zebrafish larvae and extracellular recording techniques, we present evidence for neuronal hyperexcitability following loss of Tsc1. Importantly, *tsc1* morphants show no evidence of tuber formation in histological studies. In addition, rapamycin effectively inhibited target of rapamycin (TOR) kinase activity in zebrafish with no amelioration of the hyperexcitable phenotype. Our study demonstrates that Tsc1 knockdown in a relatively simple brain structure can result in hyperexcitability.

Keywords: tuberous sclerosis complex (TSC), seizures, zebrafish, morpholino, rapamycin

INTRODUCTION

Tuberous sclerosis complex (TSC) is a devastating multi-organ disease associated with benign tumors and severe neurological manifestations. Epileptic seizures are the most common neurological problem in these patients, and are often refractory to treatment (Thiele, 2004). Although it is commonly believed that structural abnormalities in the brain are associated with TSC-related seizures (Shepherd et al., 1995; Goodman et al., 1997), the underlying mechanisms involved in seizure generation remain unknown. The majority of children with TSC develop cortical tubers, a brain lesion from which the disease was named (Gomez, 1995). Tubers are focal areas of disorganized cortical lamination containing pathological neuronal and glial cell types that likely result from abnormal cellular migration or differentiation during development (Crino, 2004; DiMario, 2004; Jozwiak and Jozwiak, 2005). While surgical tuber resection can reduce seizure frequency (Koh et al., 2000; Weiner et al., 2004), a less invasive treatment is desired.

TSC is an autosomal dominant disorder caused by mutations in either the *TSC1* or *TSC2* genes (Consortium, 1993; van Slegtenhorst et al., 1997). The *TSC1* and *TSC2* gene products, hamartin and tuberin, form a heteroduplex that regulate many cellular functions (for reviews see Hay and Sonenberg, 2004; Pan et al., 2004; Inoki et al., 2005; Crino et al., 2006). Under normal conditions, the tuberin/hamartin complex (THC) regulates intrinsic GTPase activity of Ras homologue enriched in brain (Rheb) (Tee et al., 2003) an mRNA shown to be induced by electroconvulsive seizures (Yamagata et al., 1994). THC functions as a GTPase-activator protein (GAP) that prevents Rheb from activating mammalian target of rapamycin (mTOR) kinase (Long et al., 2005). The mTOR pathway provides an attractive target for treating TSC symptoms and an available immunosuppressant drug (rapamycin) binds to a multi-protein complex that interacts with TOR to inhibit its kinase activity

(Brown et al., 1994; Sabatini et al., 1994). While rapamycin and its structural analogs are being tested as anticancer therapeutics (Dancey, 2005), the effect of rapamycin as an antiepileptic drug for controlling seizures in TSC have not been reported.

Animal models may be useful for understanding the etiology and treatment of TSC. To date, two vertebrate models of TSC have been identified or generated. The Eker rat was the first animal model identified with a germ-line mutation in the *Tsc2* gene (Yeung et al., 1994). While homozygous *Tsc2* mutant rats are not viable, heterozygous *Tsc2* (Eker) rats recapitulate some aspects of human TSC (Eker et al., 1981). Similar to Eker rats, targeted deletion of either *Tsc1* or *Tsc2* in mice results in embryonic lethality (Onda et al., 1999; Kobayashi et al., 2001). However, mice with conditional inactivation of the *Tsc1* gene in glia (i.e., *GFAP:Cre* conditional knockout mice) are viable into early adulthood. Interestingly, these mice exhibit spontaneous seizures (Uhlmann et al., 2002) and dysfunctional glia (Wong et al., 2003; Jansen et al., 2005). To assess functional consequences of TSC gene inactivation in a simple vertebrate species, we focused on zebrafish (*Danio rerio*).
Here, we present evidence for an increase in brain excitability in the absence of tuber formation, and demonstrate that rapamycin is ineffective in reducing hyperexcitability.

METHODS

Animal Care and Maintenance

Zebrafish of the TL strain were maintained according to standard procedures (Westerfield, 1995), and following guidelines approved by the University of California, San Francisco Institutional Animal Care and Use Committee. Zebrafish embryos and larvae were maintained in egg water (0.03% Instant Ocean) unless otherwise stated.

Morpholino Design and Microinjection

cDNA sequences of two zebrafish *tsc1* (*tsc1a* and *tsc1b*) and one *tsc2* were obtained by performing BLAST searches against the zebrafish genome (http://www.ensembl.org/Danio_rerio/blastview) and EST databases (http://www.ncbi.nlm.nih.gov/BLAST/). These sequences correspond to GenBank accession numbers XM_682479 (*tsc1a*) and XM_691747 (*tsc1b*), and CD014673 (*tsc2*). cDNA sequences were confirmed by RT-PCR and DNA sequencing. Morpholino oligonucleotides were designed near the junction of the 5'-untranslated region (UTR) and open reading frames (ORF) in order to block translation, according to the manufacturer suggestions (GeneTools). Morpholino sequences used for this study were: *tsc1a*, 5'-TCGATGTCCTGTGGTGTCAGCATG-3'; *tsc1b*, 5'-AGCTGTTCCCTGGACATGACGGTGT-3'; and *tsc2*, 5'-TTAACAGCAGTGAGAGAGCCGTCTG-3'; and standard control 5'-CCTCTTACCTCAGTTACAATTTATA-3'. Morpholinos were diluted to 0.25 and 0.5 mM in 1x Danieau buffer [58 mM NaCl, 0.7 mM KCl, 0.4 mM $MgSO_4$, 0.6 mM $Ca(NO_3)_2$, 5 mM HEPES, pH 7.6] and were microinjected into zebrafish embryos at the 1-2 cell stage using a Picospritzer (General Valve). Approximately one-tenth of the single-cell volume was injected as judged by 0.05% phenol red in the injection solution.

Western Blot Analysis

Control and morphant larvae (30 each) at 3 days postfertilization (dpf) were homogenized in PBS with a protease inhibitor cocktail (Roche) and 1 mM EDTA on ice using a disposable homogenizer (Kontes). SDS sample buffer was added and the samples were boiled for 5 min. After cooling to room temperature, the samples were centrifuged at 3,000 xg for 5 min to pellet insoluble material. The equivalent of one larvae per well were subjected to 7.5% SDS-PAGE. Following transfer to nitrocellulose, the blots were blocked in 5% skim milk, incubated with primary antibodies: TSC1

(1:500, Santa Cruz Biotechnology); TSC2 (1:2,000, Santa Cruz Biotechnology); S6 (1:500, Cell Signaling); PS6 (1:2,000, Cell Signaling), followed by secondary antibodies conjugated to horseradish peroxidase (Amersham), and visualized by Enhanced ChemiLuminescence Plus (Amersham).

Electrophysiology

To obtain stable physiological recordings, zebrafish larvae at 3 dpf were immobilized in 1.2% low-melting temperature agarose in zebrafish egg water. Larvae were embedded so that the dorsal aspect of the brain was accessible for electrode placement. Embedded larvae were bathed in egg water and visualized using a Leica stereo-microscope. Under direct visual guidance, a glass microelectrode (~1.2 μm tip diameter, 2-7 MΩ) was placed in the optic tectum, the largest midbrain structure in the zebrafish. A bipolar stimulating electrode (Frederick Haer & Co) was placed on the contralateral eye [for recording configuration see Fig. 2(A)]. Electrodes were filled with 2 M NaCl and electrical activity was recorded using an Axopatch 1D amplifier (Axon Instruments). Voltage records were low-pass filtered at 1-2 kHz (-3 dB, 8-pole Bessel), high-pass filtered at 0.1-0.2 Hz, digitized at 5-10 kHz using a Digidata 1300 A/D interface, and stored on a PC computer running pClamp software (Axon Instruments).

Electrophysiological recordings were analyzed *post hoc* using Clampfit software (Axon Instruments). Field excitatory postsynaptic potential (fEPSP) data was evaluated for all fish at a stimulation intensity of 1.4 mV; traces were classified as "epileptiform" if they exhibited complex multi-spike responses during the recovery phase of the fEPSP trace (e.g., 50 to 500 msec after the peak downward deflection). Spontaneous gap-free recordings, 10 minutes in duration, were analyzed for all fish. A threshold for detection of spontaneous events was set at 20 μV (peak-to-peak amplitude) and 200 msec (duration); all events exceeding these thresholds were analyzed.

Immunohistochemistry

Control and *tsc1a* morphants at 3 dpf were euthanized in tricaine (Sigma), fixed in 4% paraformaldehyde/PBS for 2 hours at room temperature, cryoprotected in 30% sucrose/PBS at 4° overnight, embedded in O.C.T. in individual 0.2 ml tubes and stored at -20°. Transverse sections (20 μm thick) were cut on a cryostat (Leica). Slides were dried at room temperature for 1 hour before processing for immunohistochemistry. Slides were washed 2 X 10 minutes in 1X PBS and incubated in blocking solution, 5% normal goat serum in 0.1% Triton X-100 in PBS (PBT), for 2 hours at room temperature. Primary antibody mouse anti-HuC/HuD (1:500, Molecular Probes) was added in blocking solution and incubated overnight at 4°. Slides were washed 3 X 15 minutes in PBT and incubated in secondary antibody Alexa Fluor 488 goat anti-mouse (1:500, Molecular Probes) for 2 hours at room temperature. Slides were then washed 3 X 15 minutes PBT and 1 X 10 minutes PBS. Slides were coverslipped with Aquamount (Lerner Laboratories) and stored at 4°. Fluorescence staining was visualized with a confocal microscope (Leica).

Semi-thin Plastic Sections

Larvae were fixed in 2.5% glutaraldehyde/1% paraformaldehyde in PBS overnight at 4^0C followed by fixation in 1% osmium tetraoxide for 1 hr at room temperature. Fixed larvae were dehydrated through a series of ethanol washes followed by 100% propylene oxide. After overnight equilibration in 1:1 propylene oxide:EMbed (47.5% EMbed 812, 33.0% DDSA, 18% NMA, 1.5% BDMA, v/v), larvae were transferred into 100% EMbed, placed into molds, and polymerized at 40^0C overnight followed by 70^0C overnight. The larvae were sectioned at 2 μm, stained with 1% tuluidine blue/1% sodium borax, and examined by light microscope.

Rapamycin Treatment

Stock solutions of rapamycin (Sigma) were prepared in DMSO (Sigma) at a concentration of 10 mM and stored in aliquots at -20^{0}C. Working concentrations (50 nM to 1 M) were made fresh in egg water. Wild-type, control-injected, and *tsc1a* morphants were treated with rapamycin 24-hours prior to Western blot and electrophysiological analysis.

RESULTS

Identification of Zebrafish *tsc* Homologs

We used a database mining approach to identify zebrafish *tsc* genes. Two *tsc1* paralogs (*tsc1a* and *tsc1b*) and one *tsc2* ortholog were identified. RT-PCR demonstrated that mRNAs for *tsc1a*, *tsc1b*, and *tsc2* genes are inherited maternally and are expressed at 3 dpf (data not shown). Both *tsc1* genes code for putative proteins showing sequence identities of 44.3% for Tsc1a and 49.7% for Tsc1b compared to the human sequence. Consistent with an ancient genome duplication in zebrafish (Postlethwait et al., 1998), Tsc1a and Tsc1b protein sequences showed only 54.4% sequence identity. By examining zebrafish and human genome sequences, we determined that *tsc1a* was located on linkage group 5 in a genomic region with a high level of synteny to human 9q34, the chromosomal location of human *TSC1* (van Slegtenhorst et al., 1997). For zebrafish *tsc2*, only the N-terminal region, corresponding to amino acids 1-138, was cloned and sequenced. This partial sequence shows 63.3% sequence identity to human tuberin.

***tsc1a* Morpholinos Knockdown Hamartin and Tuberin Expression**

Antisense morpholino oligonucleotides were used to disrupt *tsc* gene function in developing zebrafish. Morpholinos are short oligos that are comprised of a nucleic acid base, a morpholine ring, and a non-

ionic phosphorodiamidate intersubunit linkage that block protein translation by targeting the 5'UTR of mRNA (Nasevicius and Ekker, 2000). *tsc1a*, *tsc1b*, and *tsc2* morpholinos were designed by GeneTools according to recommended guidelines (www.gene-tools.com), and the effectiveness of knocking down hamartin and tuberin protein expression was analyzed using Western blot. Following injection at the 1-2 cell stage, embryos were raised to 3 dpf then hamartin and tuberin were examined. As shown in Figure 1, injection of 0.25 mM *tsc1a* morpholino reduced hamartin expression by about one-half (n = 30), and injection of 0.5 mM *tsc1a* morpholino abolished hamartin expression (shown in duplicate; n = 30 each). Tsc1a morpholinos also knocked down tuberin levels (Fig. 1), suggesting that in the absence of hamartin, tuberin is unstable and possibly targeted for degradation. In contrast, *tsc1b* morpholinos had no effect on hamartin or tuberin expression (data not shown, n = 30). Injection of the *tsc2* morpholino (0.5 mM; n = 30) knocked down tuberin levels, but had no effect on hamartin (Fig. 1). We chose to analyze morphants at 3 dpf, because morpholinos can lose their effectiveness later in development (Rinner et al., 2005), and this is the earliest time point in which "seizure-like" activity can reliably be generated in larval zebrafish (unpublished observations by M.R.T. and S.C.B.). Because *tsc1a* morpholinos produced the most significant disruption of Tsc proteins, the remaining experiments in this study use only *tsc1a* morphants.

We also examined the phosphorylation state of ribosomal protein S6 in *tsc* morphants at 3 dpf. In the absence of a functional THC complex, TOR kinase activity is up-regulated, resulting in phosphorylation of S6 (Kwiatkowski et al., 2002). Surprisingly, we did not detect elevated levels of phospho-S6 (P-S6) in *tsc1a* or *tsc2* morphants (Fig. 1). As a loading control, the level of unphosphorylated S6 was also unchanged.

Electrophysiological Analysis of *tsc1a* Morphants Reveals Hyperexcitability

To test the physiological effects of knocking down hamartin function, we performed electrical recordings in *tsc1a* morphants. A bipolar stimulating electrode was placed on the retina and an extracellular field recording electrode was placed in the contralateral optic tectum [Fig. 2(A)]. At low stimulus intensities, there was a small negative-going field excitatory postsynaptic potential (fEPSP) that reached peak amplitude within ~15 msec; higher stimulus intensities elicited larger fEPSP responses [Fig. 2(A)]. Evoked activity was abolished by addition of 1 mM kynurenic acid, a non-specific glutamate receptor antagonist, to the bathing medium (data not shown; $n = 4$). In control zebrafish larvae (3 dpf; $n = 13$), retinal stimulation at 1.4 mV elicited a simple fEPSP that decayed back to baseline within ~300 msec. Complex late-onset activity consisting of multiple fEPSP responses were observed in less than half of the control fish tested [Fig. 2(B,C)]. In contrast, at the same retinal stimulation intensity *tsc1a* morphant zebrafish larvae consistently exhibited late-onset multiple fEPSP responses i.e., evoked epileptiform burst discharge [Fig. 2(B,C); $n = 17$].

During gap-free recording of tectal activity from control-injected zebrafish larvae ($n = 12$) in normal bathing medium, brief small-amplitude burst-like discharges were infrequently observed [Fig. 3(A,B)]. In contrast, large-amplitude burst-like discharges were consistently noted in *tsc1a* morphant zebrafish larvae under the same recording conditions [Fig. 3(A,B); $n = 24$]. The duration of these complex field events was nearly 3 times that observed for spontaneous discharge in control fish [Fig. 3(D)]. Moreover, these discharges closely resemble large-amplitude "ictal-like" activity elicited during exposure to pentylenetetrazol (PTZ) in wild-type zebrafish larvae at 7 dpf (Baraban et al., 2005). At 3 dpf, large-amplitude epileptiform discharges were also observed in control zebrafish during PTZ exposure (Fig. 4). PTZ evoked spontaneous discharge amplitude and duration was similar when comparing age-matched control and *tsc1a* morphant larvae [Fig. 4(B2,3)]. However, the frequency of

PTZ elicited spontaneous discharge was significantly higher at 5 and 15 mM PTZ exposures in *tsc1a* morphants [Fig. 4(A1,2) and Fig. 4(B1)].

Absence of Tuber Formation in *tsc1a* Morphants

Next, we performed histological examinations to determine whether tubers form in the central nervous system of *tsc1a* morphants. Figure 5 shows representative immunohistological [Fig. 5(A,B)] and semi-thin [Fig. 5(C-J)] sections of control injected [Fig. 5(A,C,E,G,I)] and *tsc1a* morphants [Fig. 5(B,D,F,H,J)]. Immunofluorescent staining with the neuronal marker HuC showed no obvious differences between control injected (Fig. 6A; $n = 6$) and *tsc1a* morphants [Fig. 5(B); $n = 6$]. To examine brain and cellular morphology in more detail, we cut semi-thin plastic sections across the entire brain and show representative sections near the forebrain [Fig. 5(C,D)], midbrain [Fig. 5(E,F)], and hindbrain [Fig. 5(G-J)]. No evidence of tubers, "tuber-like" structures, or loss of lamination was observed in *tsc1a* morphants (control, $n = 6$; Tsc1a morphants, $n = 6$). Close-up images of the zebrafish brain did not reveal obvious differences in cell soma size between control injected and *tsc1a* morphant larvae [Fig. 5(I,J)].

Rapamycin Inhibits TOR Kinase Function in Zebrafish

Rapamycin blocks TOR kinase activity and is currently in pre-clinical testing for TSC (Franz et al., 2006). As an indicator of TOR function, the phosphorylation state of ribosomal protein S6 can be monitored (Kwiatkowski et al., 2002; Onda et al., 2002). To determine the effects of rapamycin in zebrafish, embryos at 2 dpf were exposed overnight to rapamycin (50-250 nM) and analyzed by immunoblotting. As shown in Figure 6A, rapamycin inhibits phosphorylation of S6 in a dose-dependent manner in zebrafish larvae. The level of unphosphorylated S6 was unaffected by rapamycin treatment [Fig. 6(A)].

Rapamycin Does Not Alleviate TSC Hyperexcitability

To test whether rapamycin alters the electrical phenotype observed in *tsc1a* morphants, zebrafish larvae were treated with 1 □M rapamycin for 24 hr. We did not observed any effect on zebrafish development or behavior with zebrafish larvae exposed to this concentration of rapamycin for 10 days (n = 30). Following rapamycin treatment, zebrafish larvae at 3 dpf were immobilized in agarose for electrophysiological recording (n = 16). Similar to non-treated controls [Fig. 2(C)], simple fEPSP responses and rare small amplitude brief duration spontaneous discharges were observed [Fig. 6(B,C)]. In contrast, *tsc1a* morphants treated with rapamycin exhibited complex epileptiform-like fEPSP activity in response to tectal stimulation and spontaneous discharge activity in gap-free recordings resembling that seen in non-treated *tsc1a* morphants [compare Fig. 2(C) and 3(A) with Fig. 6(B) and 6(C), respectively].

DISCUSSION

Zebrafish offer many advantages for the study of neurological diseases like tuberous sclerosis complex (TSC). First, zebrafish were recently described as a model to examine seizure disorders (Baraban et al., 2005; Baraban, 2006). In this previous study, we demonstrated that zebrafish larvae, exposed to a common convulsant agent, PTZ, show "interictal-" and "ictal-like" bursts, multiple behavioral seizure stages, and an increased expression of the immediate early gene *c-fos*. Second, zebrafish gene function can be easily and rapidly "knocked down" using morpholinos (Nasevicius and Ekker, 2000). Morpholinos are highly-specific antisense oligonucleotides that act by blocking translation of mRNA. Loss-of-function can be examined very early in development and up to 3-4 dpf following morpholino injection (Nasevicius and Ekker, 2000; Sollner et al., 2004; Rinner et al., 2005). Third, zebrafish are vertebrates and have organ systems very similar to that of higher organisms. Unlike invertebrate

models, such as *Drosophila* and *C. elegans*, zebrafish have a complex brain with structures similar to those found in rodents (Wullimann et al., 1996; Mueller and Wullimann, 2005). Finally, potentially therapeutic compounds can be tested by adding substrates directly to the water in which embryos develop (Goldsmith, 2004; Taylor et al., 2004). With these advantages in mind, we developed a zebrafish model of TSC.

TSC is a devastating autosomal dominant disease that results in severe neurological symptoms including mental retardation, autism, and seizures (DiMario, 2004; Jozwiak and Jozwiak, 2005; Crino et al., 2006). While defective genes have been identified, the underlying cause(s) of seizures in TSC patients are not known. From a clinical perspective, cortical tubers, for obvious anatomical reasons, have been the focus for epilepsy treatments. However, recent evidence suggests that tubers may not be the source of seizure activity in all cases. In mice with a conditional glial fibrillary acidic protein (GFAP)-driven knockout of *Tsc1*, spontaneous electrographic seizure activity was recorded, but no evidence of tubers was described (Uhlmann et al., 2002). In humans, FDG-PET imaging studies identified epileptogenic regions of hypometabolism outside tubers (Chandra et al., 2006), and video-EEG recording localized seizure onsets to non-tuberous brain regions (Wu et al., 2006). Thus, it remains unclear whether the connection between tubers and seizures is causative, correlative, or completely unrelated.

Here we showed that *tsc1a*, but not *tsc1b*, morpholinos were effective at knocking down both hamartin and tuberin protein levels in zebrafish; *tsc2* morpholinos only knocked down tuberin protein levels. These results are in agreement with previous studies showing that *Tsc1*-null mouse embryonic fibroblasts (MEFs) have reduced hamartin and tuberin expression, whereas tuberin, but not hamartin, expression is reduced in *Tsc2*-null MEFs (Zhang et al., 2003).

Next, we examined brain activity in *tsc1a* morphants using three different recording strategies. First, evoked field responses monitored in the optic tectum revealed clear evidence for synaptic hyperexcitability e.g., the presence of multiple fEPSPs. These evoked responses in the zebrafish tectum are similar in waveform to evoked field responses recorded in neocortex or hippocampus in conditions previously described as "epileptic" (Schwartzkroin and Prince, 1978; Barkai and Hasselmo, 1994; Luhmann et al., 1998; Patrylo et al., 1999; Doherty et al., 2000). Second, spontaneous events characterized as epileptiform discharge were consistently observed in *tsc1a* morphants but not age-matched controls. Again, these electrical waveforms closely resemble burst-like discharges recorded from *in vitro* or *in vivo* rodent seizure models (Oliver et al., 1977; Piredda et al., 1986; Leweke et al., 1990). Indeed, we previously demonstrated that zebrafish PTZ-induced "ictal-like" discharge can be blocked by diazepam and valproic acid e.g., conventional antiepileptic drugs (Baraban et al., 2005). In the current study, we also showed that PTZ exacerbated this hyperexcitable phenotype in *tsc1a* morphants as burst frequency was increased in morphants compared to controls at two different PTZ concentrations. Collectively, these experiments demonstrate for the first time that reduced *tsc* gene function results in neuronal hyperexcitability in zebrafish larvae.

Because cortical tubers are believed to participate in seizure generation, we performed a thorough histological examination of *tsc1a* morphant brains. Using a combination of immunohistochemistry and high-resolution serial sectioning, we were unable to detect any brain abnormalities. There are at least two possible explanations. First, it is possible that tuber formation is a lengthy process that takes months or even years to become detectable. Formation of tubers is thought to occur mainly during brain development, and have been reported at 19-20 weeks gestation (Park et al., 1997; Wei et al., 2002). Thus, it seems unlikely that tubers would form as early as 3 dpf in zebrafish larvae. Another

potential explanation is that tubers may be specific to humans and require the presence of cortical gyri. Rodent models do exhibit some features of TSC such as renal cell carcinomas, astrocytomas, and abnormal cell types reminiscent of those found in cortical tubers (Onda et al., 1999; Kobayashi et al., 2001; Uhlmann et al., 2002; Takahashi et al., 2004; Wenzel et al., 2004). However, cortical tubers have not consistently been reported in any lissencephalic rodent model examined [note one aged Eker rat was described with a brain malformation resembling a tuber (Mizuguchi et al., 2000)]. From these studies, it appears likely that cortical tubers may not be entirely necessary for seizure generation.

Currently, there is no reliable treatment for seizures in TSC patients. However, recent studies have shown some positive effects with the drug rapamycin. In the Eker rat model, rapamycin was shown to be effective in treating pituitary and renal tumors (Kenerson et al., 2005). More recently, rapamycin was shown to cause regression of astrocytomas in human TSC patients (Franz et al., 2006). However, the effect of rapamycin in treating seizures has not yet been reported. Here, we showed that rapamycin was effective in blocking TOR kinase function in zebrafish, but was not effective in reducing the hyperexcitability observed in *tsc1a* morphants. These data suggest that TSC-related seizures may not be due to up-regulation of TOR kinase activity. Interestingly, we found that TOR kinase activity was not up-regulated in *tsc1a* morphants. We expected an increase in the phosphorylation of ribosomal protein S6, because TOR function is predicted to increase with loss of *tsc* gene function (Kwiatkowski et al., 2002; Onda et al., 2002). Perhaps at this very rapid stage of development, the translational machinery is functioning near a maximum level, causing saturation of S6 phosphorylation. Because hyperexcitablity was generated without evidence of increased TOR activity, these data further support the idea that TOR function may not directly contribute to the seizure phenotype.

In conclusion, we demonstrated that loss of *tsc* gene function in developing zebrafish results in hyperexcitability (a hallmark feature of an epileptic brain) in the absence of tuber formation. We should emphasize that our results do not preclude the possibility that tubers may exacerbate seizures in TSC patients, only that a hyperexcitable phenotype can be observed in the absence of tuber formation. In addition, we provide evidence that rapamycin may not reduce the abnormal electrical activity associated with TSC-related seizures.

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FIGURE LEGENDS

Figure 1 Western blot analysis of TSC morphants. Control (0.5 mM), *tsc1a* (0.25 mM and 0.5 mM), and *tsc2* (0.5 mM) morpholinos were injected into embryos at the 1-2 cell stage and raised to 3 dpf (*n* = 30 for each group). The equivalent of one larvae per lane was probed with antibodies against hamartin (TSC1), tuberin (TSC2), phosphorylated S6 (P-S6), and S6. *tsc1a* morphants at 0.5 mM were analyzed in duplicate. Note the effectiveness of *tsc1a* morpholinos at knocking down both hamartin and tuberin levels, whereas *tsc2* knocks down only tuberin.

Figure 2 Hyperexcitability in evoked field responses. (A) Schematic showing the recording configuration for agar-immobilized zebrafish; a stimulating electrode was placed on one eye and a recording electrode was placed in contralateral optic tectum (top). Representative input-output responses for stimulations at 0.7, 1.4, and 2.1 mV in wild-type larvae are shown below. (B) Bar graph showing the percentage of all fish in which stimulation evoked responses were obtained (*n* = 30) and classified as multiple; stimulation at 1.4 mV. (C) Representative fEPSP responses from four different control zebrafish (numbers below each trace correspond to pClamp files) and four different *tsc1a* morphants. Note the prominent presence of complex multi-spike fEPSP responses in morphants but not controls.

Figure 3 Hyperexcitability under normal recording conditions. (A) Representative gap-free recording from a control fish in normal bathing medium. One brief burst-like event was observed (a, shown at high resolution on right). In contrast, *tsc1a* morphants consistently displayed complex burst-like discharge in normal bathing medium; high resolution discharge shown in b. (B) Bar graph plotting the frequency of burst-like discharges during gap-free recording in control and *tsc1a* morphants. (C) Same

for amplitude. (D) Same for duration. Data are presented as mean ± S.E.M.; $^{*}p < 0.001$ using a one-way ANOVA.

Figure 4 Hyperexcitability in the presence of a convulsant. (A) Gap-free recordings from control (A1) or Tsc1a morphant (A2) zebrafish exposed to 5 mM PTZ for 45 minutes. Below each recording are raster plots showing the frequency of burst-like discharge for six different control and six different *tsc1a* morphant larvae; 10 min recording epoch. Blocks represent burst discharges. (B) Raster plots of burst frequency for control (B1) and *tsc1a* morphant (B2) larvae exposed to 15 mM PTZ for 45 minutes. Plot in B2 shows that PTZ-evoked burst discharge amplitudes were similar. Plot in B3 shows that PTZ-evoked burst discharge durations were similar. Data are presented as mean ± S.E.M.

Figure 5 Histological analysis of control and *tsc1a* morphants. Immunostaining with the neuronal marker HuC/HuD of control-injected (A) and *tsc1a* morphant (B) larvae, and semi-thin plastic sections of control-injected (C,E,G,I) and *tsc1a* morphant (D,F,H,J) larvae are shown. No obvious differences were observed between control injected ($n = 6$) and *tsc1a* morphants ($n = 6$). Schematic representation of the location of sections [1 is shown in (C,D); 2 is shown in (A,B,E,F); 3 is shown in (G,H,I,J)] from a dorsal (K) and a lateral (L) perspective (A, anterior; P, posterior; L, left; R, right; D, dorsal; V, ventral). Scale bar shown in (H) is 50 μm in (A-H) and scale bar in (J) is 10 μm in (I and J).

Figure 6 Effect of rapamycin on TOR kinase and electrical activity. (A) Western blot analysis of rapamycin-treated zebrafish. Larvae were treated with rapamycin (0-250 nM) for 24 hours prior to analysis at 3 dpf. Rapamycin blocked the phosphorylation of the ribosomal protein S6 in a dose-dependent manner. S6 levels were unaffected by rapamycin treatment. (B,C) (B) Evoked fEPSP responses for one control and two different *tsc1a* morphants exposed to rapamycin (24 hr) are shown.

Note the similarity between Tsc1a traces here and in [Fig. (2)]. (C) Gap-free recordings of tectal activity in a control and *tsc1a* morphant zebrafish exposed to rapamycin for 24 hr. Note the similarity between Tsc1a traces here and in [Fig. 3(A)].

Figure 1

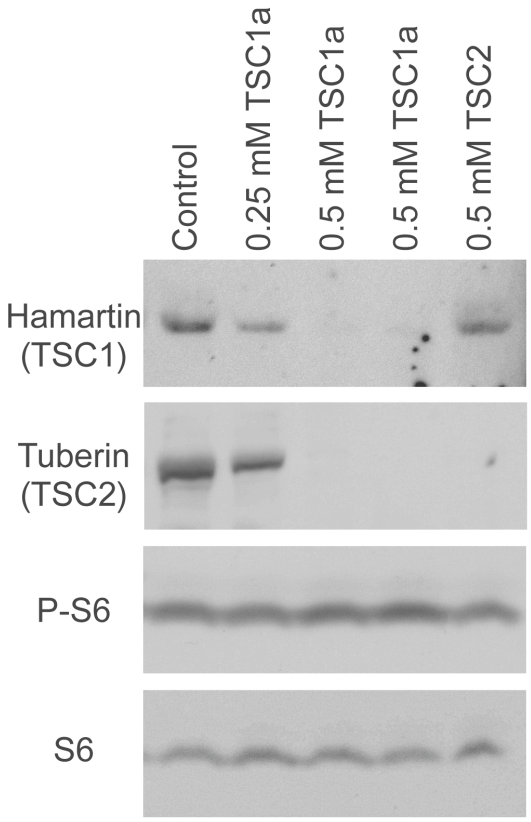

Control
0.25 mM TSC1a
0.5 mM TSC1a
0.5 mM TSC1a
0.5 mM TSC2
Hamartin (TSC1)
Tuberin (TSC2)
P-S6
S6

Figure 2

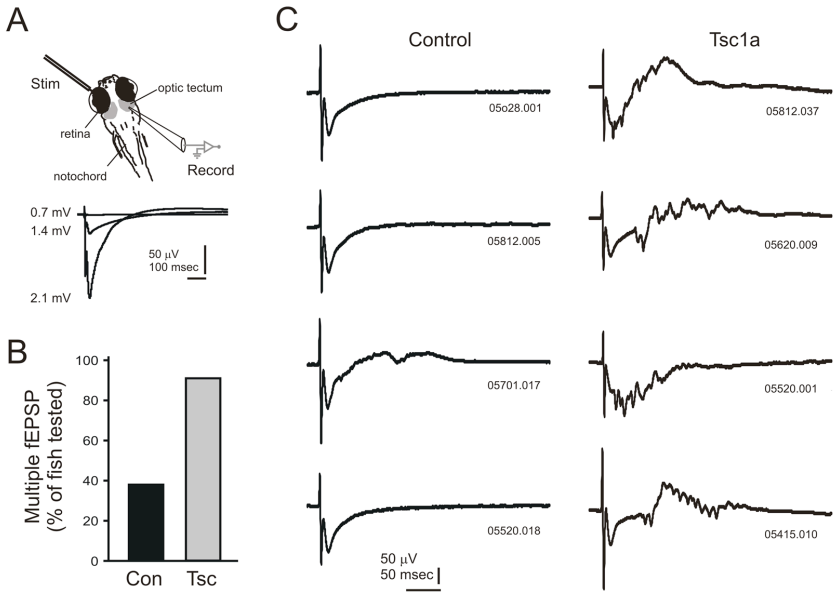
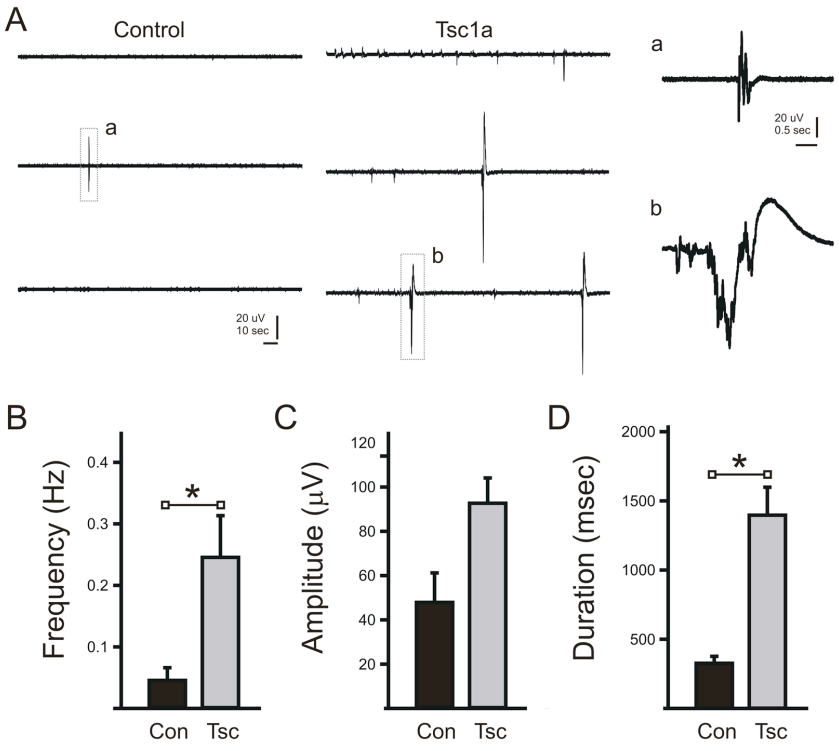

A
Stim
optic tectum
retina
notochord
Record
0.7 mV
1.4 mV
2.1 mV
50 μV
100 msec
B
Multiple fEPSP
(% of fish tested)
100
80
60
40
20
0
Con
Tsc
C
Control
Tsc1a
05o28.001
05812.037
05812.005
05620.009
05701.017
05520.001
05520.018
05415.010
50 μV
50 msec

Figure 3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Control
Tsc1a
a
b
20 uV
10 sec
20 uV
0.5 sec
B
Frequency (Hz)
0.4
0.3
0.2
0.1
*
Con
Tsc
C
Amplitude (μV)
120
100
80
60
40
20
Con
Tsc
D
Duration (msec)
2000
1500
1000
500
*
Con
Tsc

Figure 4

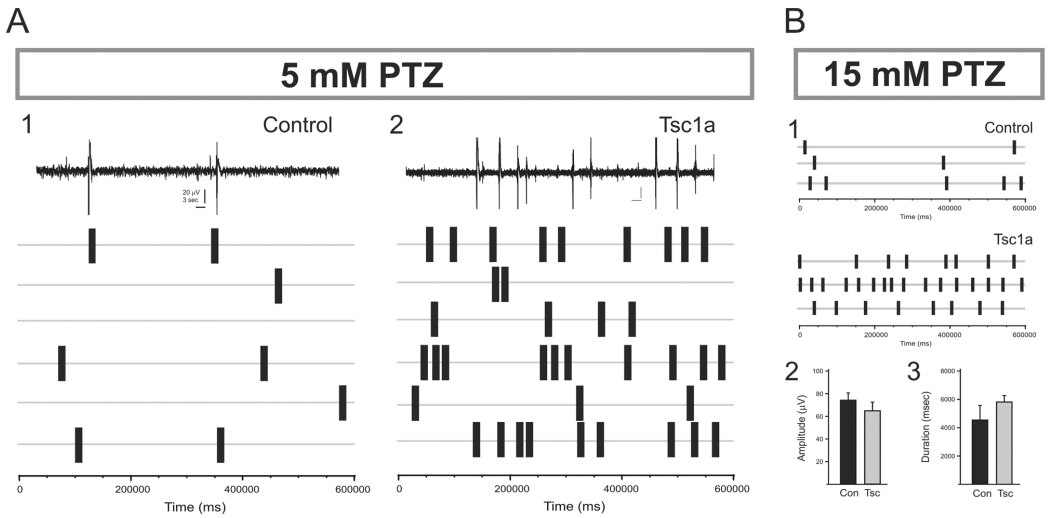

A
5 mM PTZ
1
Control
2
Tsc1a
20 μV
3 sec
0
200000
400000
600000
Time (ms)
B
15 mM PTZ
1
Control
Tsc1a
Time (ms)
2
3
Amplitude (μV)
Duration (msec)
Con
Tsc

Figure 5

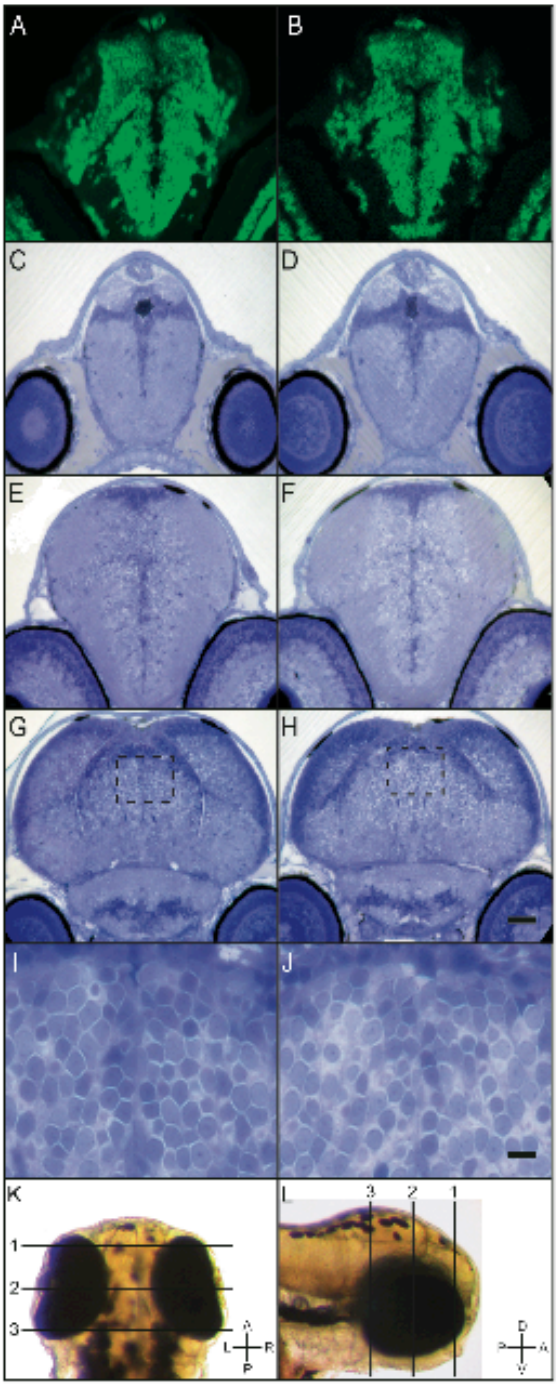
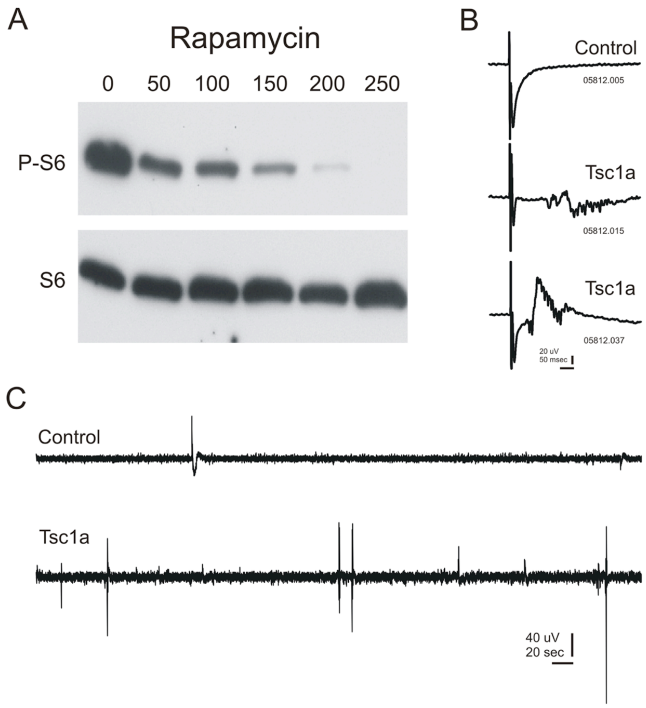


Figure 6


A
Rapamycin
0 50 100 150 200 250
P-S6
S6
B
Control
05812.005
Tsc1a
05812.015
Tsc1a
05812.037
20 uV
50 msec
C
Control
Tsc1a
40 uV
20 sec